



Troglitazone inhibits expression of the phosphoenolpyruvate carboxykinase gene by an insulin-independent mechanism

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Abstract

Troglitazone is an oral insulin-sensitizing drug used to treat patients with type 2 diabetes. A major feature of this hyperglycemic state is the presence of increased rates of hepatic gluconeogenesis, which troglitazone is able to ameliorate. In this study, we examined the molecular basis for this property of troglitazone by exploring the effects of this compound on the expression of the two genes encoding the major regulatory enzymes of gluconeogenesis, phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) in primary cultures of rat hepatocytes. Insulin is able to inhibit expression of both of these genes, which was verified in our model system. Troglitazone significantly reduced mRNA levels of PEPCK and G6Pase in rat hepatocytes isolated from normal and Zucker-diabetic rats, but to a lesser extent than that observed with insulin. Interestingly, troglitazone was unable to reduce cAMP-induced levels of PEPCK mRNA, suggesting that the molecular mechanism whereby troglitazone exerted its effects on gene expression differed from that of insulin. This was further supported by the observation that troglitazone was able to reduce PEPCK mRNA levels in the presence of the insulin signaling pathway inhibitors wortmannin, rapamycin, and PD98059. These results indicate that troglitazone can regulate the expression of specific genes in an insulin-independent manner, and that genes encoding gluconeogenic enzymes are targets for the inhibitory effects of this drug. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

Type 2 diabetes is characterized by reduced insulin sensitivity, resulting in decreased glucose metabolism and increased hepatic glucose output, as well as a

failure by the pancreas to compensate for this insulin resistance due to defects in glucose-stimulated insulin secretion (reviewed in [1]). The resulting hyperglycemia, and the inability of many patients to adequately regulate their blood glucose levels, is what eventually leads to the debilitating and sometimes fatal aspects of this disease including cardiomyopathy, retinopathy, nephropathy, and neuropathy. It is now well recognized that regulation of blood glucose levels in these patients should be the primary concern upon which their treatment is based [2].

Glucose homeostasis involves the coordinated regulation of several metabolic pathways including glu-

Abbreviations: db-cAMP, *N*⁶,2'-*O*-dibutyryl adenosine 3',5'-cyclic monophosphate; DMSO, dimethylsulfoxide; G6Pase, glucose-6-phosphatase; PEPCK, phosphoenolpyruvate carboxykinase; PPAR, peroxisome proliferator-activated receptor; RPPO, ribosomal phosphoprotein PO

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coneogenesis. Phosphoenolpyruvate carboxykinase (PEPCK) is generally considered to be the rate-limiting enzyme of this pathway [3], although glucose-6-phosphatase (G6Pase) is also a pace-setting enzyme [4]. Therefore, it is not surprising that the activity of PEPCK is elevated in nearly all models of diabetes examined to date, regardless of whether they are of chemical or genetic origin [5–8]. Indeed, an type 2 diabetes-like syndrome can be produced in rodents simply by over-expressing the PEPCK gene [9]. This observation emphasizes the role that PEPCK plays in the complications of, and in some cases perhaps the etiology of, diabetes. An interesting feature of this enzyme that distinguishes it from most other rate-limiting enzymes is that it has no known allosteric modifiers [10]. Instead, regulation of its activity is exerted primarily through control of gene expression [11]. It should be noted that two isoforms of PEPCK exist. The mitochondrial form is constitutively expressed, while the cytosolic form is tightly regulated by multiple hormones at the level of transcription, and is the form of this enzyme which is elevated in diabetes [11].

A number of oral hypoglycemic or anti-hyperglycemia agents have been developed for the treatment of type 2 diabetes, with differing mechanisms of action. The most recently developed group of compounds, which are structurally unrelated to sulfonylureas and biguanides, are the thiazolidinediones of which troglitazone is the member currently in clinical use [12]. Troglitazone improves hepatic, adipose, and skeletal muscle insulin resistance and ameliorates hyperinsulinemia in both obese–non-diabetic patients and those with type 2 diabetes (reviewed in [12,13]). It is effective in lowering serum triglycerides and raising HDL cholesterol [13]. Of most importance, however, is its ability to lower blood glucose levels which it achieves, at least in part, by inhibiting hepatic gluconeogenesis [14,15].

Since troglitazone is a relatively new drug, information regarding its molecular mechanism of action is sparse. While it is clear that it acts to potentiate the action of insulin, there is also evidence that it has direct insulin-like effects in specific tissues, including effects on gene expression [16,17]. At least some of these effects result from direct binding of thiazolidinediones to the γ -isoform of the peroxisome proliferator-activated receptors (PPARs) [18,19]. Given that

the regulation of PEPCK and G6Pase activity is primarily determined at the level of transcription, and that one of the physiological effects of thiazolidinediones is to decrease hepatic gluconeogenesis [14,15], we examined in the present study the effect of troglitazone on the expression of genes encoding these two gluconeogenic enzymes.

2. Materials and methods

2.1. Materials

Dulbecco's modified essential medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco-BRL (Burlington, Ont., Canada). Troglitazone and PD98059 were gifts from Parke-Davis (Ann Arbor, MI, USA), and metformin was obtained from Nordic (Kirkland, Ont., Canada). Wortmannin was purchased from Biomol (Plymouth Meeting, PA, USA), and rapamycin was from ICN Pharmaceuticals (Costa Mesa, CA, USA). Insulin, salmon sperm DNA, $N^6,2'$ -*O*-dibutyryl adenosine 3',5'-cyclic monophosphate (db-cAMP), and collagen were purchased from Sigma-Aldrich (Oakville, Ont., Canada). Collagenase was obtained from Worthington (Freehold, NJ, USA). Restriction endonucleases were from Promega (Madison, Wisconsin, USA). Klenow enzyme and TriZol reagent were acquired from Gibco-BRL. Chemstrips bG and 5L (blood and urine glucose analysis, respectively) and Random Priming hexanucleotide mix were from Boehringer-Mannheim (Laval, Que., Canada). Genescreen Plus membranes and [α - 32 P]dCTP (3000 Ci/mmol) were purchased from DuPont-New England Nuclear (Boston, MA, USA). All other molecular biology grade reagents were obtained from Fisher Scientific (Nepean, Ont., Canada) and BDH (Toronto, Ont., Canada).

2.2. Animals and cell culture

Principles of laboratory animal care were followed, and all protocols were approved by the University of Saskatchewan Committee on Animal Care and Supply. Male Sprague–Dawley (150–200 g), and BB/Zucker obese–diabetic (BB/ZDP) rats were obtained from Charles River (St. Constant, Que., Canada).

and the Department of Pathology, University of Massachusetts, respectively. The animals were fed ad libitum and kept under a 12-h light–dark cycle. BB/ZDP rats used in the experiments had blood glucose levels of >17 mM and glucosuria of >14 mM. Aged-matched BB/ZDR (diabetic resistant) control rats were harvested at the same time as the BB/ZDP rats. Hepatocytes were isolated by the collage-

nase perfusion method described by Cascales et al. [20]. Cell viability was determined by the Trypan blue exclusion method and was typically $>90\%$. Cells were placed into collagen-coated 100-mm culture dishes at a concentration of one to two million cells per plate and incubated in a humidified atmosphere ($5\% \text{ CO}_2$) at 37°C . After 1 h, cells were checked for attachment and the medium replaced with $10\% \text{ FBS/DMEM}$. Following a 4 h incubation period, the medium was replaced with serum-free DMEM containing 0.2% bovine serum albumin and incubated for an additional 8 h. Cells were then treated with hormones and/or modulators for 8 h as described in the figure legends. The vehicle for troglitazone was dimethylsulfoxide, and that for metformin was phosphate-buffered saline.

2.3. Plasmids and probes

The PEPCK cDNA was released from the plasmid pPCK10 [21] by *Pst*I digestion. The cDNA for the catalytic subunit of G6Pase was released from BS/G6Pase/7.1 [22] by *Hind*III/*Bam*HI digestion. The cDNA insert for ribosomal phosphoprotein PO (RPPO), used as a control probe in the Northern experiments, was excised from p36B4 [23] by *Pst*I digestion. The cDNA probes were prepared by the random priming method, using $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$ as the radiolabeled substrate, as per instructions supplied with the hexanucleotide mixture (Boehringer Mannheim).

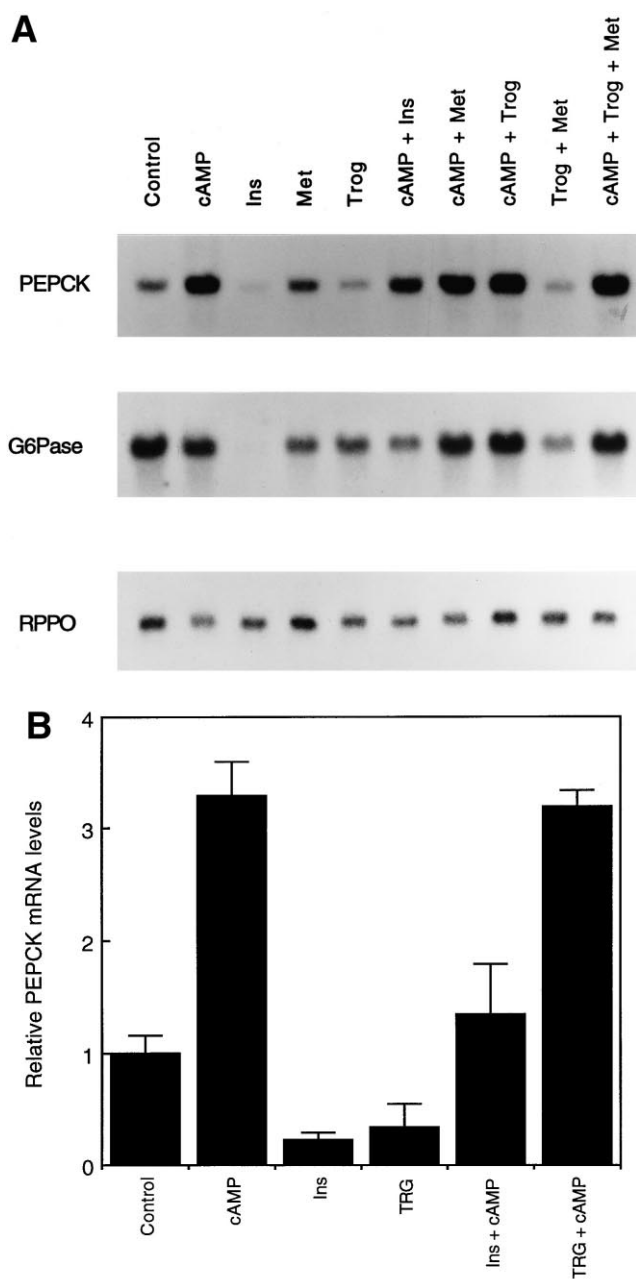


Fig. 1. Troglitazone treatment of hepatocytes leads to a reduction in PEPCK and G6Pase mRNA levels. Primary cultures of rat hepatocytes were treated for 8 h with either $500 \mu\text{M}$ db-cAMP, 10 nM insulin, $100 \mu\text{M}$ troglitazone, $250 \mu\text{M}$ metformin, or various combinations thereof. Control plates were treated with DMSO vehicle. Cells were then harvested and total cellular RNA was isolated. Northern analysis was performed on $20 \mu\text{g}$ of RNA, and the initial hybridization was performed using 10×10^6 cpm of a PEPCK cDNA probe, following by subsequent stripping and re-probing with cDNA probes for G6Pase and RPPO. The latter probe served as a control for assessment of equivalent RNA loading. Shows a representative Northern blot from a single experiment. (B) Summary of PEPCK Northern blot data (means \pm S.E.) from three experiments; only the most relevant treatments are shown. The average densitometric value for the levels in control (untreated) hepatocytes was arbitrarily set at 1.0. Ins, insulin; Trog, troglitazone; Met, metformin.

2.4. RNA extraction and Northern blot analysis

TriZol reagent was used to prepare RNA samples according to the manufacturer's instructions. For Northern analysis, 20 μg RNA were resolved on 1% agarose-formaldehyde gels and transferred onto membranes by capillary elution. Membranes were UV-crosslinked at 254 nm (1200 $\mu\text{W}/\text{cm}^2$, Stratalinker 1800, Stratagene, La Jolla, CA, USA) to facilitate subsequent stripping and reprobing. The membranes were hybridized in $5\times\text{SSPE}$ (0.75 M NaCl, 50 mM NaH_2PO_4 , 5 mM EDTA, pH 7.4), 0.5% sodium dodecyl sulfate, $5\times\text{Denhardt's}$ solution, 10% dextran sulfate containing 100 $\mu\text{g}/\text{ml}$ denatured salmon sperm DNA for 16–20 h at 65°C in a forced air rotary hybridization oven (TurboSpeed oven, Bio/Can Scientific, Mississauga, Ont., Canada). After hybridization, membranes were washed with $2\times\text{SSPE}$, 0.1% SDS at room temperature, followed by $1\times\text{SSPE}$, 0.1% SDS at 65°C . The probed membranes were exposed to X-ray film at -80°C and autoradiographs were quantified using a laser densitometer (LKB 2002 Ultrascan, Bromma, Sweden). Membranes were stripped and re-probed as per the manufacturer's instructions.

3. Results

We set out to determine whether troglitazone's ability to inhibit hepatic gluconeogenesis might be linked to an inhibition of the expression of genes encoding enzymes which regulate the rate of gluconeogenesis. PEPCK is considered to be a major pace-setting enzyme for this metabolic pathway, and alterations in PEPCK mRNA levels have been shown to accurately reflect changes in PEPCK activity as well as rates of gluconeogenesis (reviewed in [24]). As shown in Fig. 1A,B, we observed that treatment of hepatocytes with 100 μM troglitazone for 8 h decreased PEPCK mRNA levels by approximately 50% (compare Control with Trog). Insulin had a more pronounced inhibitory effect on PEPCK mRNA levels compared with troglitazone. For comparison purposes, we examined the effect of metformin, another drug used in the treatment of type 2 diabetes, for its ability to inhibit PEPCK gene expression. However, metformin, when used at concen-

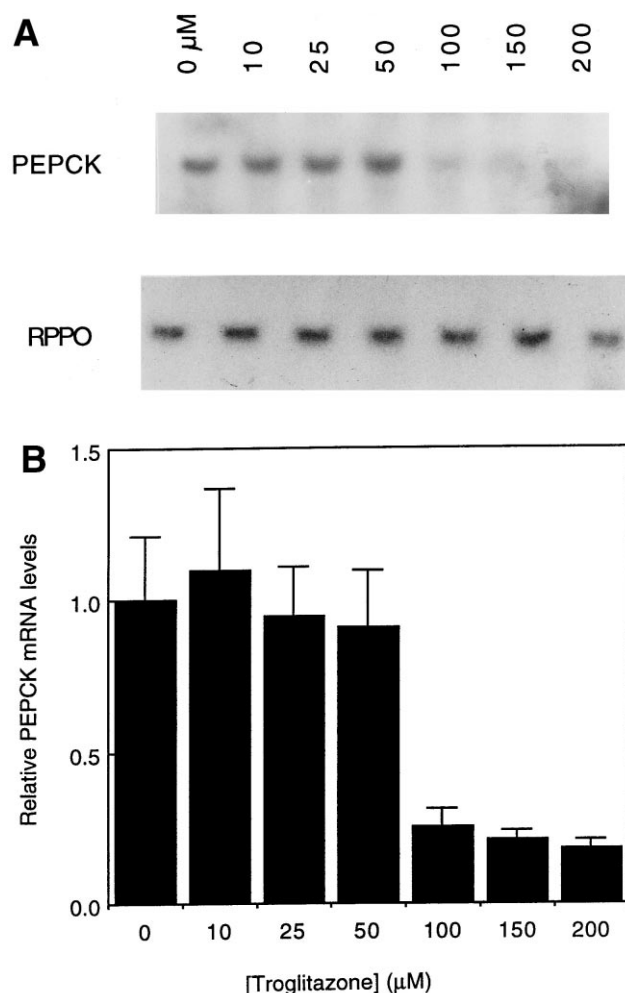


Fig. 2. Dose-response determination of the troglitazone effect on PEPCK gene expression. Primary cultures of rat hepatocytes were treated for 8 h with the indicated concentrations of troglitazone, and then RNA was isolated from the cells. (A) A representative Northern blot from a single experiment. (B) Summary of data (means \pm S.E.) from three experiments. The analysis was performed as described in the legend to Fig. 1.

trations which inhibit hepatic gluconeogenesis [25], had no significant effect on PEPCK mRNA levels (Fig. 1A). The concentration of troglitazone used in the study shown in Fig. 1 was selected based upon the dose-response experiment shown in Fig. 2, where 100 $\mu\text{mol/l}$ consistently produced an inhibitory effect. The specificity of this response produced by the relatively high concentrations of troglitazone was evident not only from the lack of effect on RPPO mRNA levels, but also from the observation that mRNA and protein levels of PPAR γ are actually induced in a dose-dependent manner by troglitazone

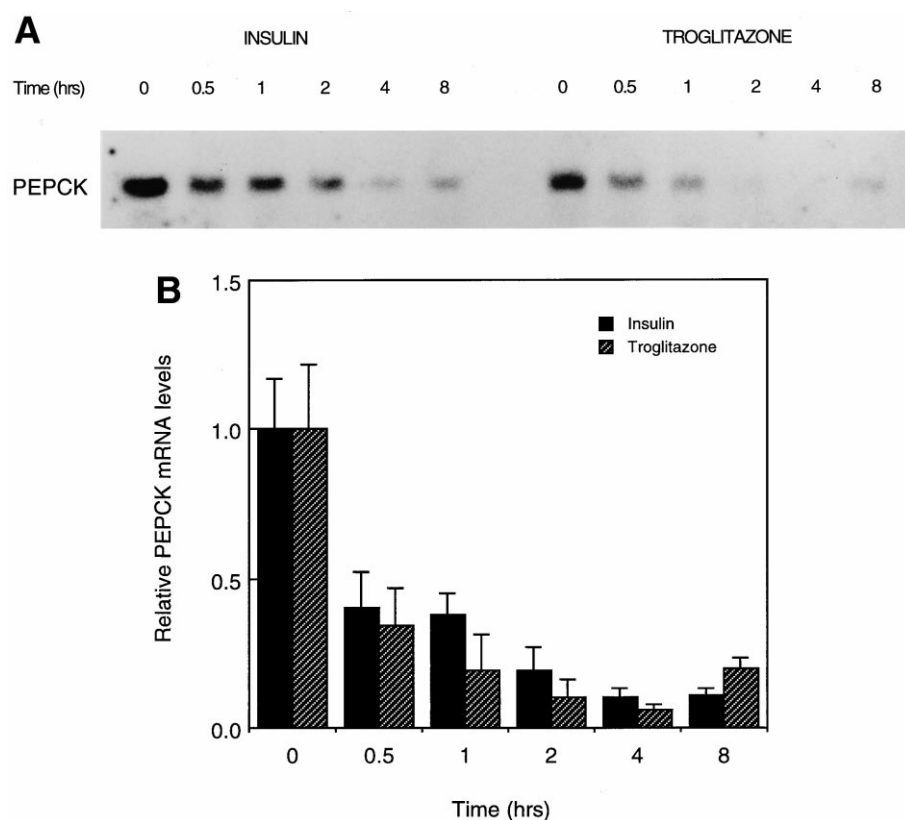


Fig. 3. Comparison of the time course of the troglitazone and insulin effect on PEPCK mRNA levels. Primary cultures of rat hepatocytes were treated with either 100 μ M troglitazone or 10 nM insulin for the indicated time, and then RNA isolated. (A) A representative Northern blot from a single experiment. (B) Summary of data (means \pm S.E.) from three experiments, with the level of mRNA at zero time being assigned an arbitrary value of 1.0. The analysis was performed as described in the legend to Fig. 1.

up to 200 μ M in isolated hepatocytes (G.F. Davies, R.L. Khandelwal, and W.J. Roesler, unpublished observations).

The transcription rate of the PEPCK gene is stimulated by cAMP, a response which can be partially or wholly reversed by insulin [26]. Since both insulin and troglitazone inhibited basal PEPCK mRNA levels, we were interested to see if troglitazone had any effect on cAMP-induced levels of PEPCK mRNA. As shown in Fig. 1A,B, db-cAMP treatment of hepatocytes led to an increase in PEPCK mRNA, and insulin treatment partially reversed this response. However, troglitazone had no effect on db-cAMP induced levels of PEPCK mRNA, suggesting that insulin and troglitazone inhibit PEPCK gene expression via different mechanisms. It should also be noted that neither metformin nor a combination of troglitazone and metformin

had any inhibitory effect on cAMP-induced mRNA levels.

G6Pase is another enzyme which is considered to catalyze a rate-limiting step in gluconeogenesis, given its position as the terminal enzyme in the pathway [4]. Since the regulation of the expression of the G6Pase gene is in some respects similar to that of PEPCK [22], we decided to examine the effect of troglitazone on its mRNA levels. Insulin had a strong repressive effect on the abundance of G6Pase mRNA (Fig. 1A), consistent with previous observations [22]. Troglitazone also led to an approximately 50% inhibition of basal G6Pase expression. In this particular experiment, metformin had an inhibitory effect, although this was not a consistent observation in the several experiments we performed (data not shown). Also evident from Fig. 1A is that while db-cAMP had no detectable ability to increase

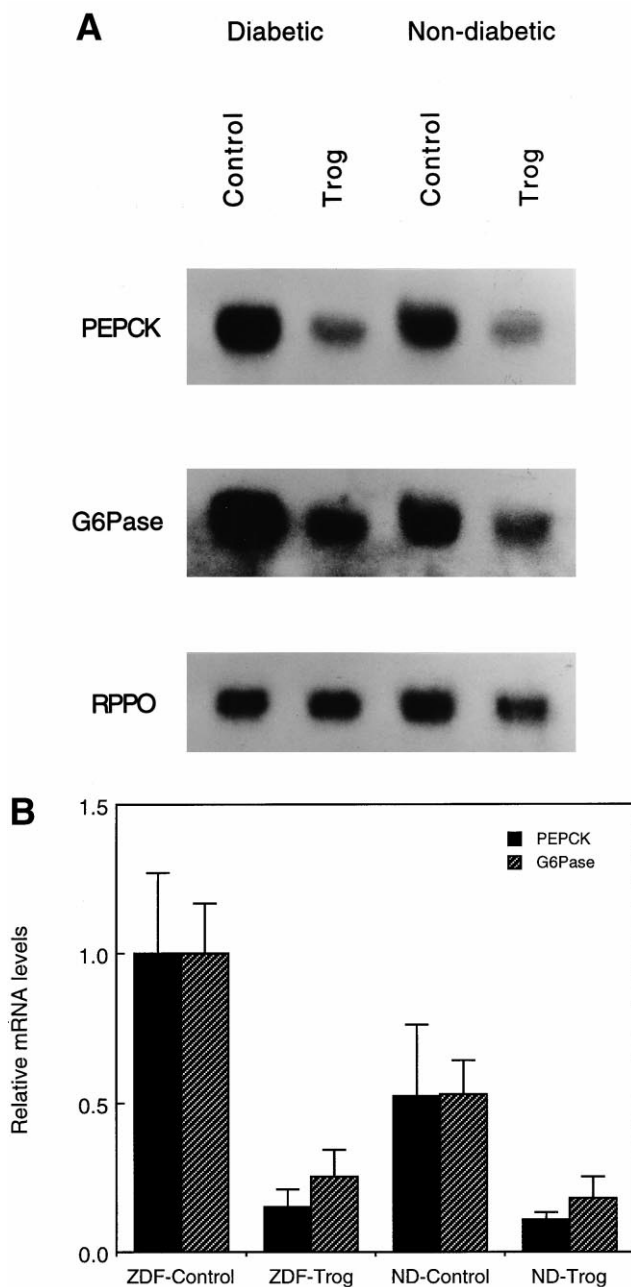


Fig. 4. Troglitazone reduces PEPCK and G6Pase mRNA levels in hepatocytes from Zucker diabetic-obese rats. Northern analysis was performed on RNA isolated from hepatocytes from Zucker diabetic-obese rats and non-diabetic littermates as described in the legend to Fig. 1. Troglitazone treatment (100 μ M) was for 8 h. (A) A representative Northern blot from a single experiment. (B) Summary of data (means \pm S.E.) from three experiments, with the level of PEPCK or G6Pase mRNA in untreated hepatocytes from diabetic rats assigned an arbitrary value of 1.0. Control plates were treated with DMSO vehicle only. ZDF, Zucker diabetic-obese hepatocytes; ND, non-diabetic hepatocytes.

G6Pase mRNA, db-cAMP did prevent the troglitazone-mediated decrease in mRNA levels.

Fig. 3A,B shows the time-course effects of troglitazone and insulin on PEPCK mRNA levels in isolated hepatocytes. Inhibition by either treatment was detectable by 30 min. However, troglitazone produced a more rapid decline in PEPCK mRNA compared with insulin, with almost undetectable levels by 2 h. At 8 h, there is a reappearance of PEPCK transcript; this is consistent with the only partial inhibition of PEPCK gene expression by troglitazone which we observed in 8-h incubation experiments (Fig. 1B).

Since troglitazone is effective in lowering blood glucose levels and hepatic glucose output in type 2 diabetes, we examined whether it could reduce the expression of PEPCK and G6Pase genes in hepatocytes isolated from the Zucker diabetic-obese rat, which are known to be insulin-resistant [27]. As shown in Fig. 4A,B, the basal levels of both PEPCK and G6Pase mRNA are elevated in these diabetic rats compared with their non-diabetic litter mates. In both non-diabetic and diabetic rats, troglitazone was able to reduce the expression of the two gluconeogenic genes. It should be noted that despite the insulin-resistant phenotype, insulin was effective in reducing PEPCK and G6Pase mRNA levels in hepatocytes prepared from these type 2 diabetic-obese rats (data not shown).

The data above suggested that troglitazone inhibits expression of the PEPCK gene by an insulin-independent mechanism, based upon the fact that there was no insulin present in the defined culture medium, and that insulin, but not troglitazone, was able to inhibit cAMP-induction of PEPCK gene expression. To test this hypothesis further, we examined the inhibitory effect of troglitazone in the presence of insulin signaling pathway inhibitors. The inhibitors used were wortmannin, which inhibits phosphatidylinositol kinase; PD98059, which binds to and inhibits MEK; and rapamycin, which is an inhibitor of p70^{s6k}. As can be observed in Fig. 5A,B, neither PD98059 nor rapamycin alone had any effect on PEPCK mRNA levels, while wortmannin treatment led to a reduction in mRNA. None of the three inhibitors tested were able to prevent the reduction in PEPCK mRNA mediated by troglitazone (Fig. 5). Consistent with previous observations [28], wortman-

nin was able to partially inhibit the insulin-mediated reduction in PEPCK mRNA (Fig. 6A,B). It should be noted that PD98059 and rapamycin have been shown previously not to block insulin's ability to inhibit PEPCK gene expression [28,29].

4. Discussion

Troglitazone is a relatively new therapeutic agent used in the treatment of type 2 diabetes. It is an

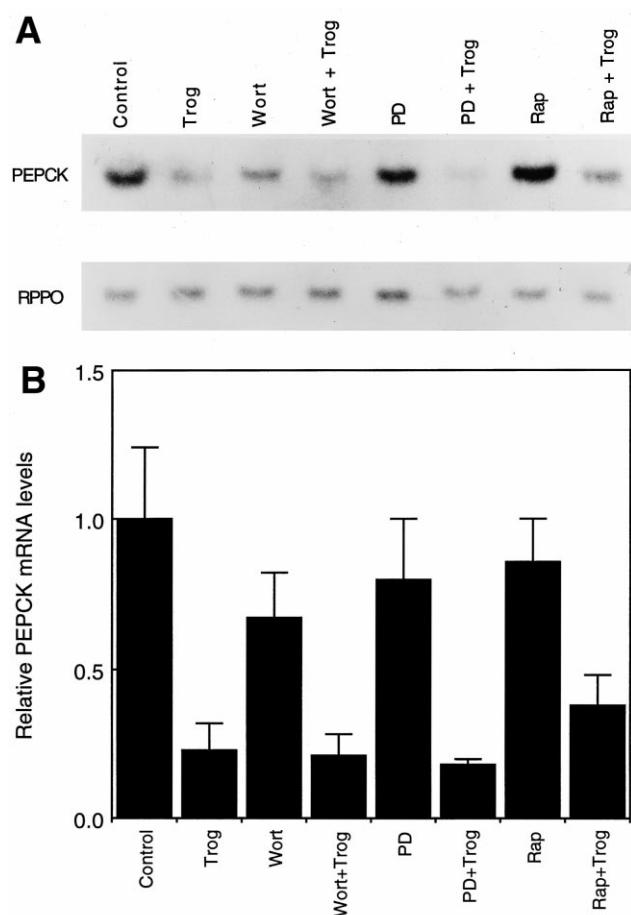


Fig. 5. Effect of insulin signaling pathway inhibitors on troglitazone-mediated inhibition of PEPCK mRNA levels. Northern analysis was performed on RNA isolated from hepatocytes from male Sprague–Dawley rats as described in the legend to Fig. 1. Hepatocytes were pretreated with wortmannin (500 nM), PD98059 (20 μ M) or rapamycin (200 nM) for 30 min prior to the addition of 100 μ M troglitazone to the media. (A) A representative Northern blot from a single experiment. (B) Summary of data (means \pm S.E.) from three experiments. Trogl, troglitazone; Wort, wortmannin; PD, PD98059; Rap, rapamycin.

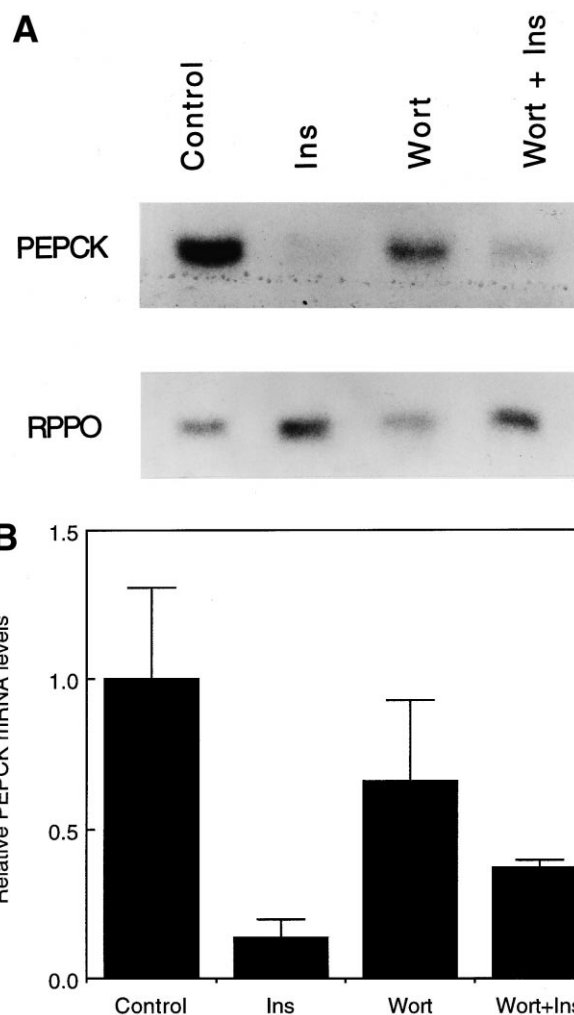


Fig. 6. Effect of wortmannin on insulin-mediated inhibition of PEPCK gene expression. Northern analysis was performed on RNA isolated from hepatocytes from male Sprague–Dawley rats as described in the legend to Fig. 1. Hepatocytes were pretreated with 500 nM wortmannin for 30 min prior to the addition of 10 nM insulin. (A) A representative Northern blot from a single experiment. (B) Summary of data (means \pm S.E.) from three experiments. Ins, insulin; Wort, wortmannin.

insulin sensitizer, and has been shown to be able to prevent or reverse several physiological alterations which are characteristic of insulin resistance [12,13]. One of the major physiological effects it has is to lower blood glucose, apparently by both inhibiting hepatic gluconeogenesis [14,15,26] and increasing glucose utilization [14,30]. While some of these effects result from an improvement in insulin sensitivity, a number of studies, including the present one, have shown that troglitazone also has direct, insuli-

nomimetic properties, including both activation and inhibition of gene expression [14,16,17,31–33].

The molecular details whereby troglitazone inhibits hepatic gluconeogenesis have not been extensively studied. Troglitazone was shown to decrease the activity of hepatic fructose-1,6-bisphosphatase in treated db/db mice [15]. It has also been postulated that troglitazone may decrease cofactor (acetyl CoA, NADH, ATP) availability which could lead to decreased rates of gluconeogenesis [34]. In the present study, we suggest an additional mechanism of action for troglitazone, that being to rapidly inhibit the expression of genes encoding two rate-limiting gluconeogenic enzymes, PEPCK and G6Pase. This effect was insulin-independent, observable in the absence of insulin in the culture medium and insensitive to insulin-signaling inhibitors. These data suggest that inhibition of PEPCK gene transcription can occur through at least two different signaling mechanisms, one being a wortmannin-sensitive insulin-dependent pathway [28] and another which is insulin-independent.

It is now documented that thiazolidinediones, like troglitazone, can lead to changes in gene expression by specifically binding to and activating PPAR γ [18,19,35]. While we have no evidence as to whether troglitazone inhibits PEPCK gene expression via a direct mechanism, the fact that the PEPCK promoter has two PPAR response elements [36] makes this a reasonable hypothesis. PPAR γ has been shown to transactivate the PEPCK promoter in non-liver cells [36]; the hypothesis we have developed suggests that in liver cells, PPAR γ , upon activation by troglitazone, inhibits the transcriptional activity of the PEPCK promoter. Precisely how the same transcription factor might confer opposite transcriptional responses in different tissues (i.e. up in adipose and down in liver) is not immediately obvious, but it may result from unique interactions of different arrays of transcription factors which bind to the promoter in select tissues. Indeed, differential regulation of the PEPCK gene in different cell types is not without precedent; glucocorticoids inhibit PEPCK promoter activity in adipocytes, but induce promoter activity in liver cells [11]. It is also possible that troglitazone indirectly inhibits PEPCK gene expression through a mechanism, for example, whereby troglitazone inhibits the expression of a transcription fac-

tor which is necessary for PEPCK gene activity in liver. The issue of whether a direct or indirect mechanism is involved is currently being addressed by examining which *cis*-element(s) in the PEPCK mediates the troglitazone response. If a direct mechanism is employed, then the troglitazone response should be mediated through one or both of the PPAR response elements.

It is also possible that troglitazone has intracellular targets in addition to PPAR γ that have not yet been identified. The possibility of a second target is in fact suggested by numerous studies showing that the biochemical and/or physiological effects of troglitazone can be grouped into two general categories, based upon whether they are observed at low or high concentrations of the drug. Many of these effects, such as stimulation of glucose uptake in adipocytes [37], upregulation of LDL receptor activity in HepG2 cells [38], stimulation of insulin release from isolated pancreatic islets [39], and inhibition of lipoprotein lipase activity in adipocytes [40], are observed at low micromolar/high nanomolar concentrations, which is consistent with the binding affinity of troglitazone to PPAR γ [41]. However, there have been reported another set of troglitazone effects, including inhibition of fatty acid oxidation and gluconeogenesis in hepatocytes [34,42] and attenuation of vascular contractility [43], which can only be observed at concentrations ranging between 30 and 100 μ M. Indeed, the degree of inhibition of fatty acid oxidation and gluconeogenesis in hepatocytes increased as troglitazone concentration was raised from 100 μ M to 1 mM [34]. It is interesting to note that in hepatocytes, inhibition of both gluconeogenesis [34] and expression of the gene which codes for the rate-limiting enzyme of this pathway (this article) require troglitazone concentrations in the higher range. Finally, it should be noted that exposure of liver to concentrations approaching and indeed exceeding 100 μ M is achievable given the current recommended therapeutic dose of troglitazone which ranges between 200 and 600 mg/day [44].

An interesting observation in the present study is that troglitazone did not interfere with cAMP-inducible expression of the PEPCK gene, another effect of this drug that distinguishes itself from that of insulin. This suggests that under troglitazone-treated conditions, the PEPCK gene remains competent for

activation for those situations when gluconeogenesis is required. This may explain one of the beneficial characteristics of this drug as being able to lower blood glucose without risk of hypoglycemia; at the first appearance of hypoglycemia, endogenous physiological signals, such as glucagon secretion, would lead to the activation of PEPCK gene expression and allow enhancement of the gluconeogenic pathway.

In summary, we have uncovered two gene targets for troglitazone which help further define the molecular mechanisms whereby it reduces hepatic gluconeogenesis and thereby ameliorates hyperglycemia. Since insulin and troglitazone appear to inhibit the expression of both of these genes by different mechanisms, this study provides further support for pursuing clinical trials to examine the benefits of combined insulin and thiazolidinedione therapy.

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